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The human cancer cell active toxin Cry41Aa from *Bacillus thuringiensis* acts like its insecticidal counterparts.

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Understanding how certain protein toxins from the normally insecticidal bacterium *Bacillus thuringiensis* target human cell lines has implications for both the risk assessment of products containing these toxins and potentially for cancer therapy. This understanding requires knowledge of whether the human cell active toxins work by the same mechanism as their insecticidal counterparts or by alternative ones. The *B. thuringiensis* Cry41Aa (also known as Parasporin3) toxin is structurally related to the toxins synthesized by commercially produced transgenic insect-resistant plants, with the notable exception of an additional C-terminal beta-trefoil ricin domain. To better understand its mechanism of action we developed an efficient expression system for the toxin and created mutations in regions potentially involved in the toxic mechanism. Deletion of the ricin domain did not significantly affect the activity of the toxin against the human HepG2 cell line suggesting that this region was not responsible for the mammalian specificity of Cry41Aa. Various biochemical assays suggested that unlike some other human cell active toxins from *B. thuringiensis* Cry41Aa did not induce apoptosis but that its mechanism of action was consistent with that of a pore-forming toxin. The toxin induced a rapid and significant decrease in metabolic activity. ATP depletion, cell swelling, and membrane damage were also observed. An exposed loop region believed to be involved in receptor binding of insecticidal Cry toxins was shown to be important for the activity of Cry41Aa against HepG2 cells.

Abbreviations

ATP, adenosine triphosphate; bp, base pair; BSA, bovine serum albumin; Bt, *Bacillus thuringiensis*; DIC, differential interference contrast; EC50, half maximal effective concentration; EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton, LB, lysogeny broth; ORF, open reading frame; PEG, polyethylene glycol; ROS, reactive oxygen species.

Introduction

Bacillus thuringiensis (*Bt*) is best known as an entomopathogenic bacterium used commercially to control various insects and nematodes (1). The main active ingredients of *Bt* based products are proteins known as Cry toxins. These are pore-forming toxins that target the epithelial cells of the insect gut following ingestion, and lead to the death of the organism (2). The genes encoding these toxins have been expressed in different crop species to produce insect resistant transgenic varieties (3). To date around 300 different Cry toxin genes have been cloned from *B. thuringiensis* (4). Although most of these toxins are active against insects, a subset, known as parasporins, have been shown to have activity against human cell lines (5). Interestingly a variety of different toxicity mechanisms have been proposed for this group of toxins. These include cell death induced by pore-formation or via a calcium-dependent apoptotic signalling pathway (5). There are currently six different classes of parasporin toxin (6) of which three (PS1, 3 and 6) are particularly interesting since the toxins are structurally very similar to those expressed by insect-resistant transgenic crops. These parasporins contain some, or all, of the five conserved sequence blocks that are a characteristic of many Cry toxins (5) and are believed to adopt a three domain structure similar to GM-crop expressed toxins such as Cry1Ac. The focus of this study was the PS3 toxin (Cry41Aa), a split toxin encoded by two separate ORFS. The gene encoding Cry41Aa is found within a three gene operon with ORF2 encoding the functional portion of the toxin and ORF3 a protein believed to be important for expression within *Bt*. The first gene (ORF1) has no known function. Cry41Aa ORF2 contains an additional domain at its C-terminus which is not found in insecticidal toxins such as Cry1Ac (7). This domain (pfam14200 ricin type beta trefoil lectin-like domain) has been implicated in binding to carbohydrates on a cell surface (8) and so could confer particular specificities to the Cry41Aa toxin. Toxins isolated from a strain (A1462) encoding both Cry41Aa and the related Cry41Ab were found to be toxic to two human cell lines (HepG2 and HL60) but non-toxic to other cell lines including HeLa (7). Importantly toxicity was only observed after proteolytic treatment of the toxins. Proteolytic activation is believed to be a crucial step in the mechanism of action of many bacterial toxins (9) including *B. thuringiensis* Cry toxins (3). The aim of this study was to build on previous work that had demonstrated the activity of Cry41Aa on HepG2 cells by investigating whether or not the ricin domain has a role to play in specificity determination, and by clarifying the molecular basis of the toxicity mechanism.

Experimental

Cloning of *cry41Aa* with and without its ricin domain

Two PCR primers were designed to amplify ORF2 and ORF3 from the *cry41Aa* operon and incorporate suitable restriction sites. The forward primer (GAAGAAGGATCCAAATGGAATGGAGG) incorporated a *Bam*HI site (underlined) 22bp upstream from the ORF2 start codon whereas the reverse primer (CGCTTCTAGAAGCCTGCTACCATTAC) incorporated an *Xba*I site 16bp downstream of ORF3. These primers allowed the two genes, and their ribosome binding sites, to be cloned into the *Bt* expression vector pSVP27a (10) using *Bam*HI and *Xba*I. This vector places cloned genes under the control of the promoter for the *Bt* Cyt1Aa toxin gene. The resulting vector (pSVP2741Aa) was introduced into the acrystalliferous *Bt* strain 4D7 obtained from the Bacillus Genetic Stock Center. To remove the ricin domain primers were designed to amplify the entire toxin encoding plasmid but excluding the ricin domain. The forward primer (TAAAGGTGTGCAACTATCCCTTGAC) included the stop codon of ORF2 at its 5' end whereas the 5' end of the reverse primer (TCGAGTGGTTAAGCCAATACCC) encoded the 713th codon of ORF2. Following amplification the PCR product was self-ligated to give a plasmid (pSVP2741AaΔricin) encoding the Cry41Aa toxin with amino acids 714-825 deleted. This plasmid was also introduced into *Bt* strain 4D7. Whole plasmid PCR was also used to create the single amino acid substitutions. All constructs were verified by DNA sequencing.

Toxin expression and purification

Recombinant *Bacillus thuringiensis* strains were grown on LB plates containing 5µg/ml chloramphenicol for 3 days at 30ºC. Cells containing spores and protoxin crystals were sonicated in water and then washed twice in 0.3M NaCl before a final resuspension in water. The protoxin was solubilized in 50mM Na_2CO_3 buffer pH 10.5 / 5mM DTT at 37ºC for one hour. Trypsin was added to the supernatant at a concentration of 1mg trypsin per mg protoxin and the sample incubated for a further hour at 37ºC. Protease inhibitor (Roche complete mini EDTA-free) was added before the activated toxin was purified on a 15ml Sephacryl S-200 High Resolution (Amersham) gel filtration column. Fractions were eluted in 25mM Tris/150mM NaCl (pH 7.4) and analysed by 7.5% SDS-PAGE and their concentration determined by the Bradford method using a Bio-Rad Protein Assay Kit using BSA as the standard.

Cell culture

The human hepatocellular carcinoma cell line (HepG2) was purchased from the European Collection of Cell Cultures (ECCAC; Salisbury, UK). HeLa cells came from an existing lab stock. Both cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 292 µg/mL L-glutamine at 37°C in a humidified 5% CO_2 incubator. When the cells reached 70-80% confluency they were split using 0.05% Trypsin-EDTA.

Cell assays

Cell viability was assessed using the Promega CellTiter-Blue assay as an endpoint method with a 2 hour incubation with the assay reagent. ATP levels were measured using Promega CellTiter-Glo. Analysis of apoptosis was performed using Promega Caspase-Glo 3/7. Membrane integrity was determined with Promega CellTox Green

assay using 'Express, No-Step Addition at Seeding Method' and Promega CytoTox-Glo. Hydrogen peroxide production was tested with Promega ROS-Glo H_2O_2 assay in the presence of 1mM sodium pyruvate. All cell assay kits were used according to the manufacturer's instructions. Where applicable, data were normalised for background fluorescence/luminescence. 90 µl of cell suspension (containing 22500 cells, unless otherwise specified) were dispensed in triplicate into a 96-well plate. After 20 hour incubation each well received 10µl of the appropriate control/test substance. Unless otherwise specified purified, trypsin-activated toxins were used in all assays. The data shown are representative results of independently repeated experiments each involving 3 technical replicates, error bars represent one standard error of the mean.

Results

Expression of recombinant Cry41Aa

The *cry41Aa* gene is found as part of a three gene operon in *Bt* strain A1462 in which the second ORF encodes the active toxin whilst the third one encodes a protein believed to aid crystallization and expression. Initial experiments confirmed that when expressed in the acrystalliferous *Bt* strain 4D7 both ORFs were expressed with apparent molecular weights of 107kDa (ORF3) and 78kDa (ORF2) (Fig 1, lane 1). Following deletion of the ricin domain, SDS PAGE analysis showed expression of both the truncated ORF2 (66kDa) and full length ORF3 as expected (Fig. 1, lane 2). Solubilization and proteolytic activation of the recombinant toxins resulted in proteins slightly smaller (by around 4kDa) than their respective unprocessed ORF2 toxins (Fig. 1 lanes 5&6). The ORF3 protein was degraded by trypsin.

Effect of the recombinant toxins on the viability of HepG2cells

The solubilised protoxin form of Cry41Aa, even at a high dose (300 µg/ml), had little effect on the viability of HepG2 cells as assessed using the CellTiter Blue cell viability assay (Fig. 2A). In contrast, activated toxin reduced viability by almost 50% at a concentration of only 10 µg/ml and was significantly more active than the mock control (unpaired t-test $P<0.005$). There was no significant difference (unpaired t-test $P>0.05$) between the ricin deleted form of Cry41Aa and the non-modified form indicating that the ricin domain does not mediate the toxic effects of Cry41Aa on HepG2 cells. Control samples included buffer alone and trypsin-treated proteins from an alternative recombinant strain of *B. thuringiensis* - 4D7 expressing the insecticidal Cry1Ca toxin. Neither of these showed any toxicity towards HepG2 cells. As previously reported (11) HeLa cells were not sensitive to protease-activated Cry41Aa (Fig. 2B). The effect of the toxin on HepG2 cells was relatively quick since maximum toxicity was observed following 2 hours of exposure to the toxin (data not shown). Since we found no evidence of any difference in activity between the full length and ricin-deleted forms of the toxin subsequent studies to probe the mechanism of action of the toxin were only performed on the full-length toxin. In order to establish an EC50 value for Cry41Aa, a dose response experiment was performed (Fig. 2C). Probit analysis generated an EC50 value of 2.17µg/ml (95% CL 1.96-2.41).

Mechanism of action of Cry41Aa on HepG2 cells

Analysis of another parasporin toxin (Cry31Aa) that shares the same toxin protein fold as Cry41Aa indicated that this toxin induced apoptosis in susceptible HeLa cells (12). We therefore investigated whether Cry41Aa's effects

on HepG2 cells were mediated by necrosis or apoptosis. Since necrotic and apoptotic processes can often be distinguished by intracellular ATP levels (13), we measured ATP levels following toxin exposure using the CellTitre-Glo assay. This showed that Cry41Aa induced a rapid reduction in ATP levels suggesting a necrotic pathway (Fig. 3A). To further test whether or not apoptosis was being induced by Cry41Aa in HepG2 cells, the levels of apoptotic mediators (Caspase 3/7) were assessed using the Caspase-Glo 3/7 Assay. Fig. 3B shows that whilst the known apoptosis-inducing drug etoposide (14) resulted in an elevation of caspase levels, Cry41Aa had no such effect suggesting that it was not acting through an apoptotic pathway. Necrotic pathways within a cell are often associated with increases in reactive oxygen species (ROS) (15), including some induced by bacterial pore-forming toxins (16), and so we investigated whether or not Cry41Aa induced an increase in H_2O_2 in HepG2 cells using the ROS-Glo assay. The results (Fig. 4) show that there was no significant increase in ROS levels when compared to the positive control, menadione.

To further investigate the mechanism of action of this toxin, its effect on the integrity of the HepG2 cell membrane was assessed using the CellTox Green cytotoxicity assay. Fig. 5A compares the effect of Cry41Aa with that of a positive control – a digitonin-based lysis solution (Promega G182B). A significant effect was seen with Cry41Aa after 90 minutes with the response after 300 minutes reaching the level seen with the positive control. After 300 minutes the signal from cells treated with 100µg/ml etoposide or Cry1Ca remained at basal level (data not shown). A further assay (CytoTox-Glo) that also assesses membrane integrity supports the breakdown of the cell membrane (Fig 5B). The above data are consistent with the toxin causing cell death via the formation of pores resulting in cell swelling and eventually lysis. To investigate the size of the pores believed to be formed by the toxin various osmoprotectants were added to cells alongside the toxin and the CytoTox-Glo assay used to measure cell integrity. Fig. 6 shows that while the monosaccharides glucose and glucosamine failed to prevent the action of the toxin, the disaccharides and larger molecules of PEG did. This result is consistent with the toxin forming discrete pores rather than having a more general detergent-like action. Microscopic analysis of treated and untreated cells showed that Cry41Aa caused swelling of the HepG2 cells within an hour of toxin addition (Fig 7).

Creation of putative receptor-binding mutants

The above data are all consistent with Cry41Aa acting as a pore-forming toxin - the same mechanism used by insecticidal Cry toxins that share the same protein fold. With the insecticidal Cry toxins, various exposed loops have been identified as being important in the interaction between the toxin and the target cell. Loop3 of domain II in particular is believed to have an important role in the initial binding to cell surface receptors (3). A candidate region (503 VRDNCPFAWPGYKQL 517) analogous to loop 3 was identified in Cry41Aa through sequence alignments with various insecticidal toxins whose structure has been solved experimentally (Cry1Aa, Cry3Aa, Cry3Bb, Cry4Aa, Cry4Ba, Cry5Ba). An homology model of the structure of Cry41Aa was created using Phyre2 (17) (Fig. 8). The model is consistent with the above region being a loop at the apex of domain II. Since aromatic residues in this putative loop have previously been shown to be important for toxicity in other toxins (18) the three aromatic residues (F509, W511 and Y514) were individually converted to alanine to assess their role in HepG2 cell killing. The three mutant toxins behaved exactly the same as the non-mutant form through expression, solubilisation, activation and purification suggesting that there had not been any significant effect on protein folding. All the toxins were tested for toxicity towards HepG2 cells using the CellTiter-Blue assay (Table 1). Of

the three mutants only Y514A retained toxicity, in contrast F509A and W511A had completely lost it. To further investigate the importance of the F509 amino acid additional substitutions were made in which the amino acid was replaced by a variety of both aromatic and non-aromatic ones. F509Y retained toxicity whereas F509W, F509S and F509L were inactive. For W511, substitutions to phenylalanine and tyrosine were created but only the former expressed well, this W511F mutant retained toxicity. Thus of the substitutions made only F509Y and W511F retained activity supporting the hypothesis that aromatic amino acids in this region are important for toxicity.

Discussion

Of all the *Bacillus thuringiensis* Cry toxins with activity against human cell lines, the Cry41A class (parasporin 3) most closely resembles their insecticidal counterparts. Sequence comparisons reveal that Cry41A contains the five highly conserved sequence blocks characteristic of the Cry toxins that are believed to possess the same three-domain structure (5). A key difference between these toxins is the presence of a ricin domain in Cry41A. This domain has been observed in many unrelated proteins and it has been proposed that it adds carbohydrate binding functionality (8). Several other toxins contain this domain including ricin (19), the HA33 component of the botulinum neurotoxin complex (20) and a large mosquitocidal toxin from *Lysinibacillus sphaericus* (21). Additionally, several other toxins from *B. thuringiensis* contain this domain: Cyt1Ca (22); Cyt1Da (23); Cry42Aa (24) and to a lesser extent the Cry35 family (25), although there is no evidence that the domain is involved in their toxicity. In contrast when this ricin domain was artificially fused to the C-terminus of the insecticidal Cry1Ac toxin the hybrid protein was found to be toxic to an increased range of insects (26). More recently this domain has been found in a 54kDa protein from *B. thuringiensis* subsp. *israelensis* (27). This latter protein is believed to have a function in stabilising a matrix surrounding the Cry toxins in this strain, potentially by interacting with carbohydrate residues. Unlike Cry41Aa, the ricin domain-containing, and apoptosis-inducing, toxin pierisin from the cabbage butterfly *Pieris rapae* is capable of killing HeLa cells (28). Mutagenesis studies suggested that the conserved QxW sequences in this domain were essential for toxicity, and were most likely involved in binding to glycosphingolipid receptors on the cell surface. In summary the ricin type beta-trefoil lectin like domain is widely found in *B. thuringiensis*, has carbohydrate binding functionality, and can help determine the specificity of a toxin.

We tested the hypothesis that this additional domain could be responsible for the human cell toxicity associated with Cry41Aa by expressing recombinant clones with and without this domain and comparing their activities on a susceptible cell line. Expression of the two components of the Cry41Aa split toxin in a *B. thuringiensis* expression system resulted in two major bands. ORF2, which encodes the active portion of the toxin, has a predicted molecular weight of 93kDa but runs as a 78kDa protein in our gel system. Previously this toxin was reported as having a mobility on SDS PAGE corresponding to an 88kDa protein (7). ORF3 has a predicted molecular weight of 82kDa but in our system runs as a 107kDa protein. This is consistent with a previous report (7) in which ORF3 migrated at 120kDa. Cry toxins are generally believed to require proteolytic activation before they exert their toxic effect, and Yamashita *et al.* indeed found that solubilised Cry41Aa was not toxic to HepG2 cells whereas toxin treated with proteinase K (giving a 64kDa fragment) was (7). Traditionally Cry toxins have been activated *in vitro* using trypsin. When we treated our recombinant Cry41Aa toxin with trypsin we did not get a 64kDa fragment but instead one that was only marginally smaller than the unprocessed toxin. We did however confirm that the trypsin-activated toxin showed activity against HepG2, whilst even a large excess of

unprocessed toxin had little activity. Upon treatment with trypsin (or proteinase K (7)) no ORF3 can be recovered suggesting that this protein does not possess a protease-resistant core. Even upon solubilisation, the ratio of ORF3 to ORF2 drops suggesting that this protein is unstable. ORF3 resembles the C-terminal half of the larger Cry toxins, a region that is believed to be important for the expression and crystallization of the toxin within *B. thuringiensis*. This region is degraded when treated with proteases and plays no further part in the toxic mechanism (29). It seems likely then that ORF3 has a similar role to this C-terminal extension despite being expressed as a separate protein. When we attempted to express ORF2 in the absence of ORF3 a protein was detected at the correct size on SDS PAGE but the toxin could neither be solubilised nor activated with trypsin, suggesting that it had either not folded or not crystallized properly (data not shown).

Deletion of the ricin domain resulted in expression of both ORF2 and ORF3 but with ORF2 migrating at 66kDa as a result of the deletion of the 12kDa domain. Both ORFs solubilized in alkali and when treated with trypsin a stable band of ORF2, at the predicted size, was obtained. Deletion of the ricin domain does not therefore appear to have significantly altered the physical properties of this protein. When tested against HepG2 cells the deleted form had a similar toxicity to the unmodified toxin indicating that the ricin domain is not crucial for HepG2 toxicity. Our data are therefore consistent with a model in which both specificity and toxicity are determined by the core three-domain protein conserved between Cry41Aa and its insecticidal counterparts.

Our data are also consistent with Cry41Aa killing HepG2 cells through a pore formation mechanism, i.e. in the same way that its insecticidal homologues are believed to kill their host cells. Rapid swelling was microscopically observed in susceptible cells, analogous to insect cells losing the ability to regulate osmotic pressure after exposure to Cry toxins (30).

Experiments with osmoprotectants were used to determine the size of the primary lesions. Assays conducted at 3 hours (data not shown) and 6 hours post treatment produced results consistent with a pore diameter of <1 nm. This is smaller than previously estimated for some Cry toxins. For Cry1C the maximum radius was estimated at between 1 - 1.3 nm (31) and for Cry1Ac 1.2 – 1.3 nm (32). However previous experiments were performed in planar lipid bilayers in the absence of receptors or with membrane vesicles devoid of cellular content, and at a relatively high pH. Other experiments based on the Trypan blue exclusion method in *C. fumiferana* cells resulted in the detection of smaller pores. Channel radii induced by *Bt kurstaki* P1 toxin were less than 0.6 nm and for *Bt aizawai* and *israelensis* toxins between 0.6 - 1.0 nm (30). CyaA a hemolysin from *B. pertussis*, is also believed to form pores of 0.6 - 0.8 nm in diameter (33).

Cry41Aa significantly affected cell viability within the first few hours, with an EC50 similar to a previous estimation (7). As with insecticidal Cry toxins, proteolytic activation of Cry41Aa was necessary for toxicity. Protoxin caused a small decrease in viability most likely due to activation by endogenous proteases since this was reduced when crystals were solubilised in the presence of protease inhibitors (data not shown). HepG2 exposure to Cry41Aa resulted in the cell membrane rapidly becoming permeable allowing the detection of two different cytotoxic markers. This effect was time and dose dependent and within 5 hours cytotoxic levels reached that caused by membrane permeabilizing agents. Toxin-induced ATP depletion, decrease in metabolic activity, swelling, and membrane damage followed a similar time course. Cry41Aa did not induce activation of caspases or oxidative stress even at low doses. All of the above data are consistent with a model in which Cry41Aa kills HepG2 cells through pore-formation followed by ROS independent necrosis. All these effects were cell and toxin specific. The structurally related three domain Cry1Ca toxin expressed and purified in the same way as Cry41Aa

did not affect HepG2 cells, and neither were HeLa cells affected by either Cry41Aa or Cry1Ca confirming the previously observed specificity (7).

How these toxins could have evolved to target certain human cells remains a mystery and it is most likely that this activity does not represent their primary target. However understanding how these toxins target human cells is important when assessing the risk of the large scale use of new insecticidal toxins. Although we have shown that the specificity/toxicity of both types of toxin (insecticidal and human-active) may be mediated through the same region, ie loop3 of domain 2, there is currently not enough understanding of the nature of this interaction to be able to predict specificity on the basis of toxin sequence or structure. Furthermore previous work has shown that new specificities can be acquired through minor changes in the loop3 sequence (34). It is clear that considerably more work needs to be done, for example in fully understanding the nature of the interaction with the host cell, in order to understand how *Bt* toxins target particular hosts, even very dissimilar ones.

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Declarations of interest

The authors declare that there are no competing interests associated with this manuscript

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Author contribution

V.K cloned and expressed the toxin, created the ricin domain deletion mutant and performed the initial Cell TitreBlue assays (Figs 1 & 2). B.D performed the other assays. A.E. created and assayed the loop mutants. F.A. created the constructs that facilitated the formation of the various mutants. M.J.W. provided expert guidance on cell culture and proofread the manuscript. N.C. provided overall academic guidance and wrote the manuscript.

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Toxin	Toxicity
Cry41Aa	Control (Yes)
F509A	No
F509S	No
F509L	No
F509W	No
F509Y	Yes
W511A	No
W511F	Yes
Y514A	Yes

Table 1. Activity of single amino acid substitution mutants towards HepG2 cells. Recombinant toxins were tested at a concentration of 12µg/ml and assayed using CellTiter-Blue. They were considered toxic if their activity was not significantly different to the non-modified toxin. They were considered non-toxic if their activity was not significantly different to the buffer control.

Figure Legends

Figure 1. Solubilization and activation of the Cry41Aa toxins. Lanes 1, 3 and 5 contain the unmodified protoxin and lanes 2, 4 and 6 contain the ricin domain deleted derivative. Lanes 1 and 2: unprocessed protoxin; lanes 3 and 4: alkali solubilized protoxins; lanes 5 and 6: trypsin-activated toxins. M: molecular weight markers (in kDa).

Figure 2. Toxicity of recombinant Cry41Aa. A) HepG2 cells were seeded at a density of 5000 cells per well and treated with either solubilised or trypsin-activated Cry41Aa, or trypsin-activated Cry41AaΔricin for 20 hours and cell viability assessed using the CellTiter-Blue assay. B) Trypsin-activiated Cry41Aa and Cry1Ca were used to treat either HepG2 or HeLa cells for 20 hours and cell viability assessed using the CellTiter-Blue assay. Etoposide (100µg/ml) and Triton X-100 (0.1%) were used as positive controls and buffer alone as a negative control. C) Cells were treated with the different toxin concentrations shown for 20 hours prior to CellTiter-Blue assay. In each assay percentage viability was calculated relative to a buffer only mock control and the error bars represent one standard error of the mean.

Figure 3. Assessment of ATP and Caspase 3/7 levels in HepG2 cells after exposure to Cry41Aa. A) HepG2 cells were dosed with Cry41Aa (12 µg/ml) or buffer. Luminescence was measured at different time points using the CellTiter-Glo assay B) Caspase levels were measured using the Caspoase-Glo 3/7 assay following exposure to the stated concentrations of toxin.

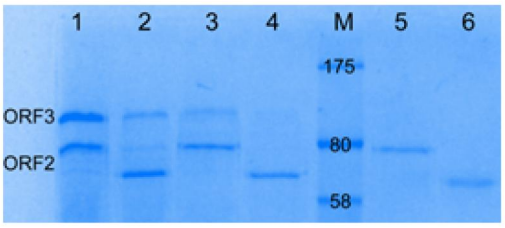
Figure 4. Estimation of hydrogen peroxide levels in HepG2 cells following treatment with Cry41Aa. HepG2 cells were dosed with H_2O_2 substrate and incubated with Cry41Aa (10 µg/ml), Menadione (50 µM), Cry1Ca (10 µg/ml), digitonin (13.1 µg/ml) or buffer only for the indicated amount of time.

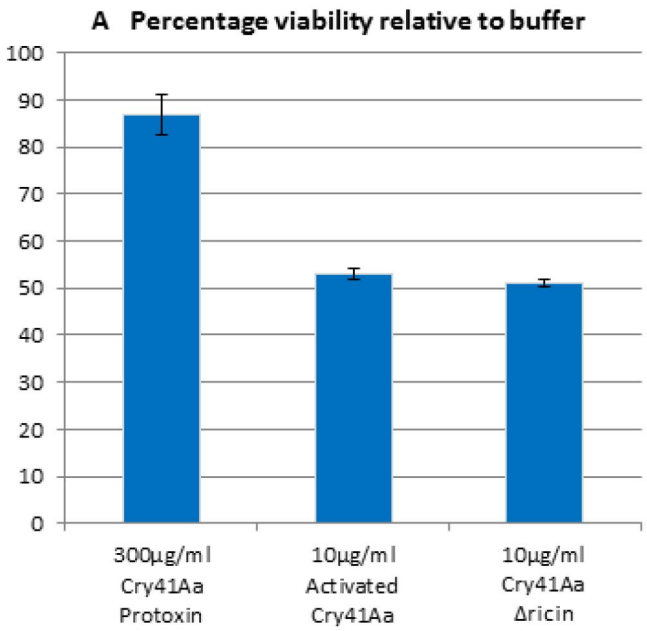
Figure 5. Assessment of the effect of Cry41Aa on the integrity of HepG2 cell membranes. A) HepG2 cells were treated with either 12µg/ml Cry41Aa, buffer only or a digitonin-based lysis solution for the times indicated and membrane integrity measured using the CellTox Green assay B) HepG2 cells were treated with either 5µg/ml Cry41Aa, 5 and 58 µg/ml Cry41Aa protoxin, 5 and 80 µg/ml Cry1Ca, buffer only or the detergent Triton X100 solution for the times indicated and membrane integrity measured using the CytoTox-Glo assay. Damage is indicated relative to cells treated with 43.7 µg/ml digitonin.

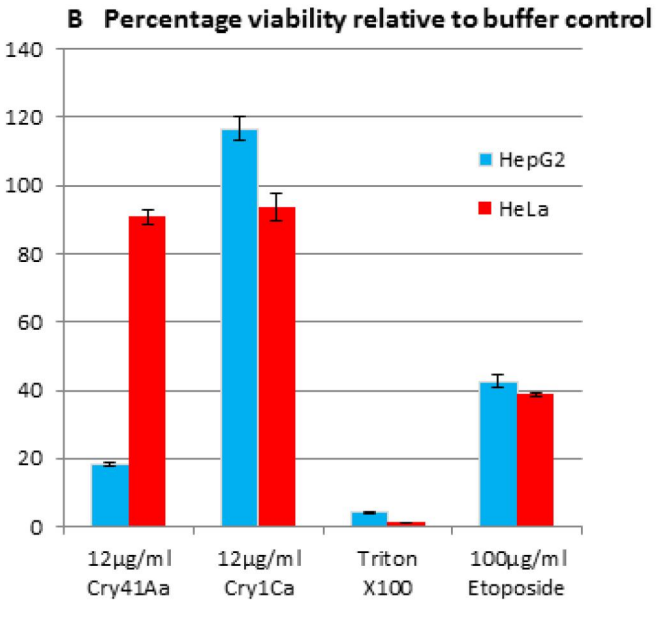
Figure 6. The effect of different osmoprotectants on HepG2 membrane permeability 6 hours after exposure to Cry41Aa. Cells were treated with various osmoprotectants (30 mM) or mock (dH_2O) for 10 minutes. This was followed by the addition of either Cry41Aa (55 µg/ml) or buffer. Luminescent readings were taken 6 hours later using CytoTox-Glo cell assay. All data were plotted as the percentage of digitonin (11.4 µg/ml) treated cells.

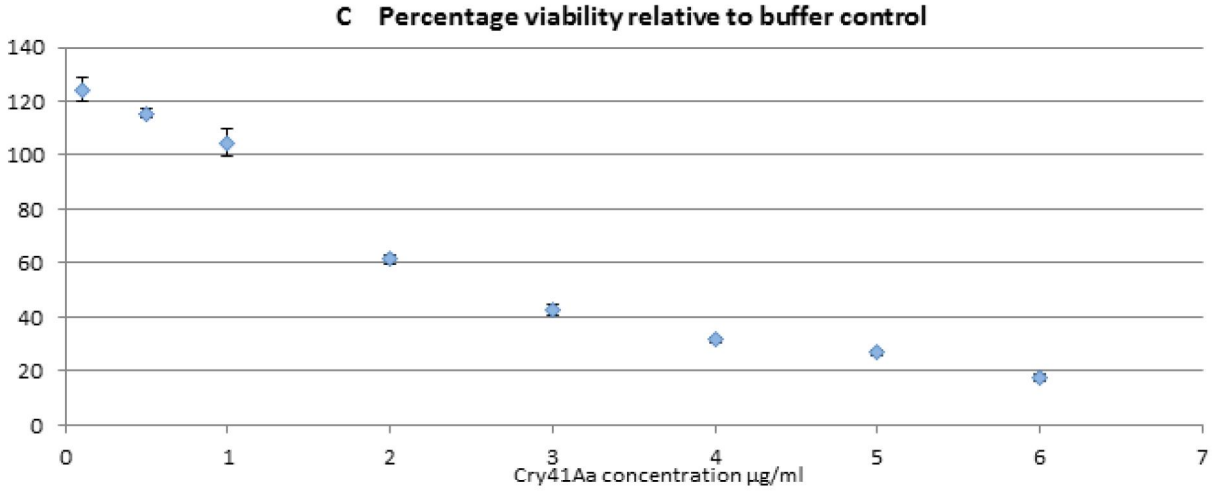
Figure 7. Effect of Cry41Aa on HepG2 morphology. HepG2 cells (1.5×10^4 per chamber) were treated with 5.5µg/ml Cry41Aa for the times indicated and then viewed by DIC microscopy. The scale bar represents 20µm.

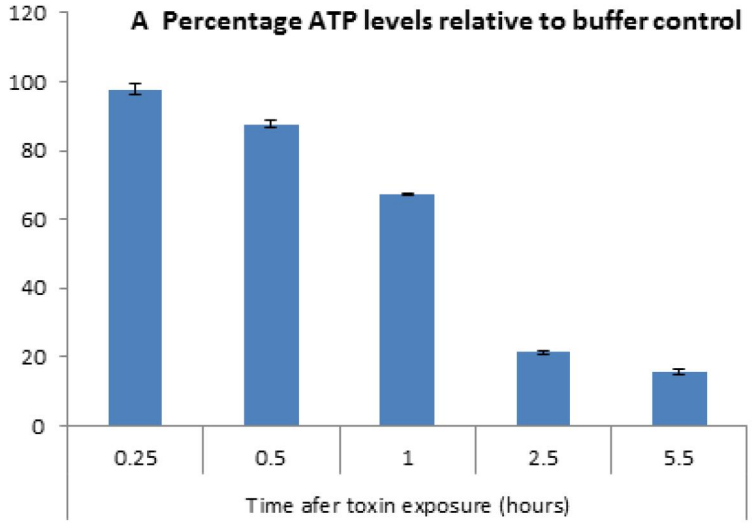
Figure 8. Homology model of Cry41Aa. The model was created using Phyre2 and displayed using Chimera1.8. Amino acids F509 and W511 are highlighted.

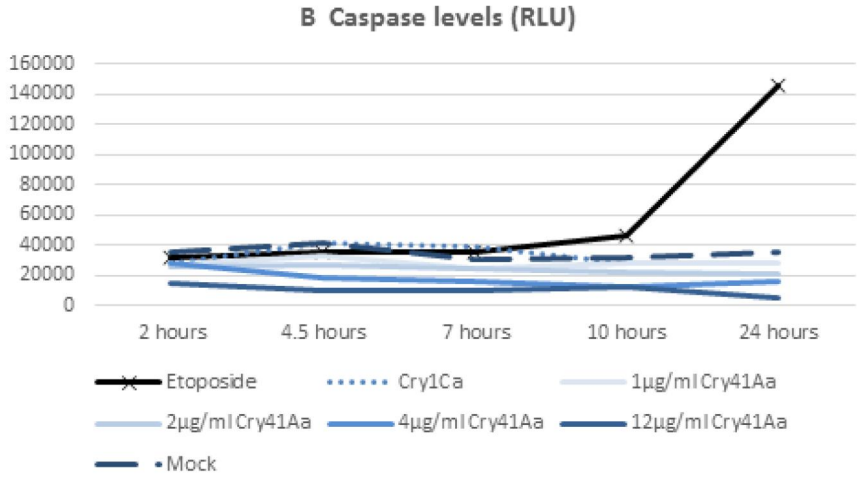

1
2
3
4
M
5
6
175
80
58
ORF3
ORF2

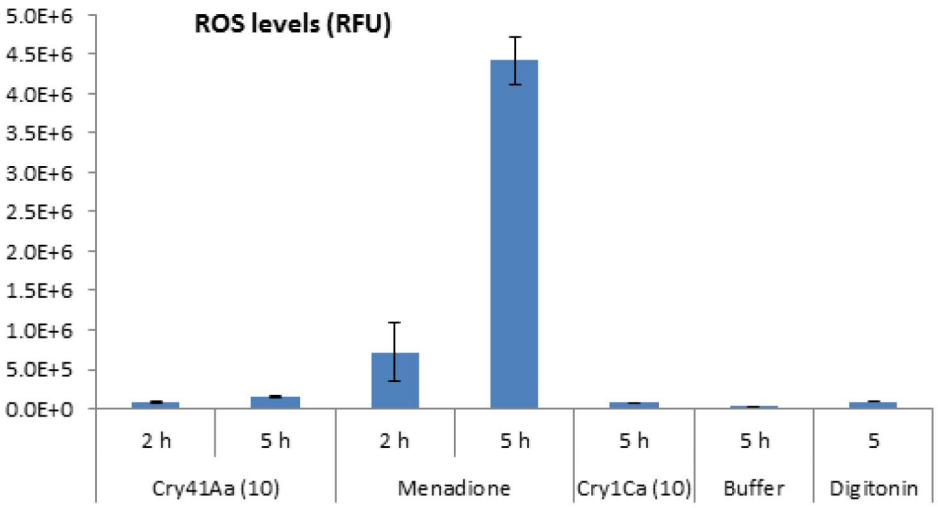

A Percentage viability relative to buffer
100
90
80
70
60
50
40
30
20
10
0
300µg/ml Cry41Aa Protoxin
10µg/ml Activated Cry41Aa
10µg/ml Cry41Aa Δricin

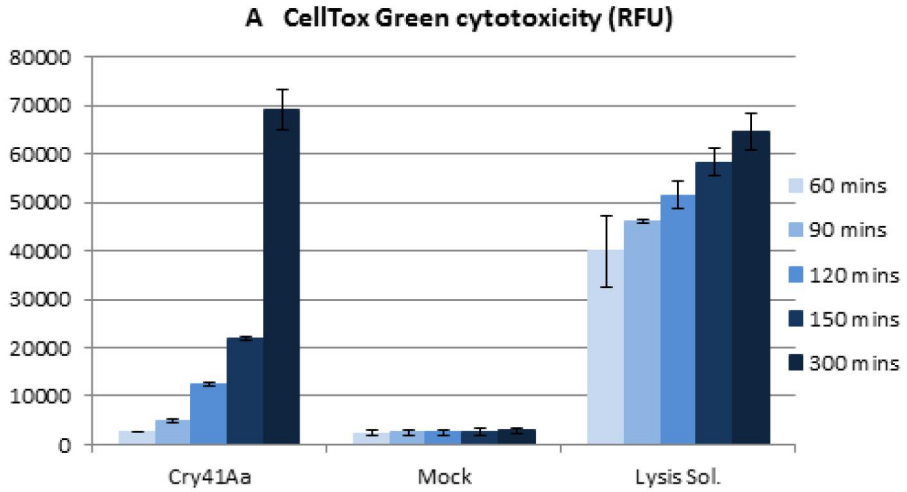

B Percentage viability relative to buffer control
140
120
100
80
60
40
20
0
HepG2
HeLa
12µg/ml Cry41Aa
12µg/ml Cry1Ca
Triton X100
100µg/ml Etoposide

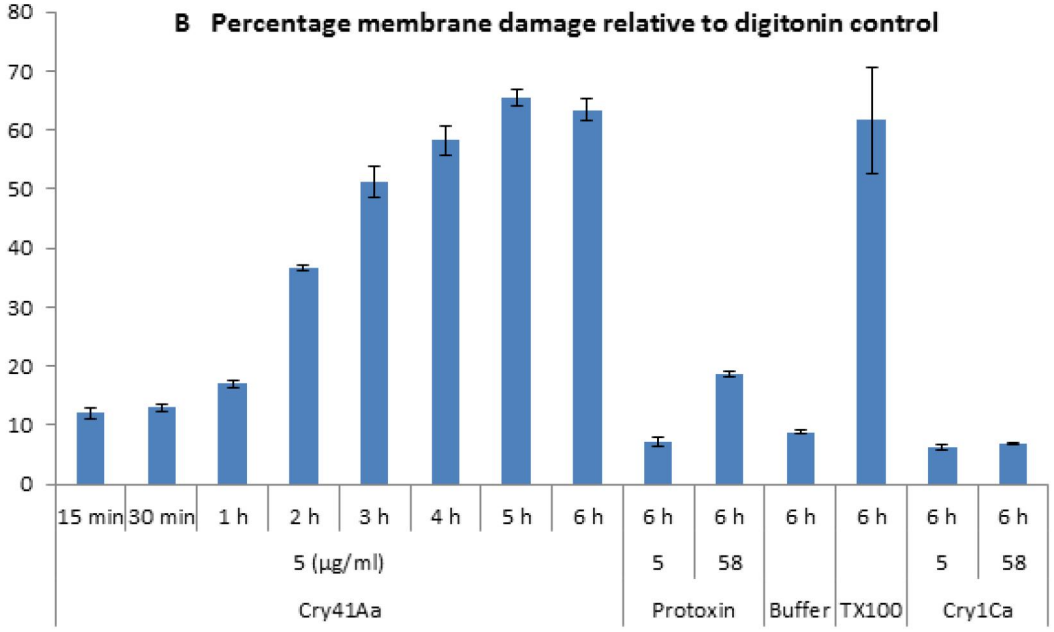

C Percentage viability relative to buffer control
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
Cry41Aa concentration µg/ml

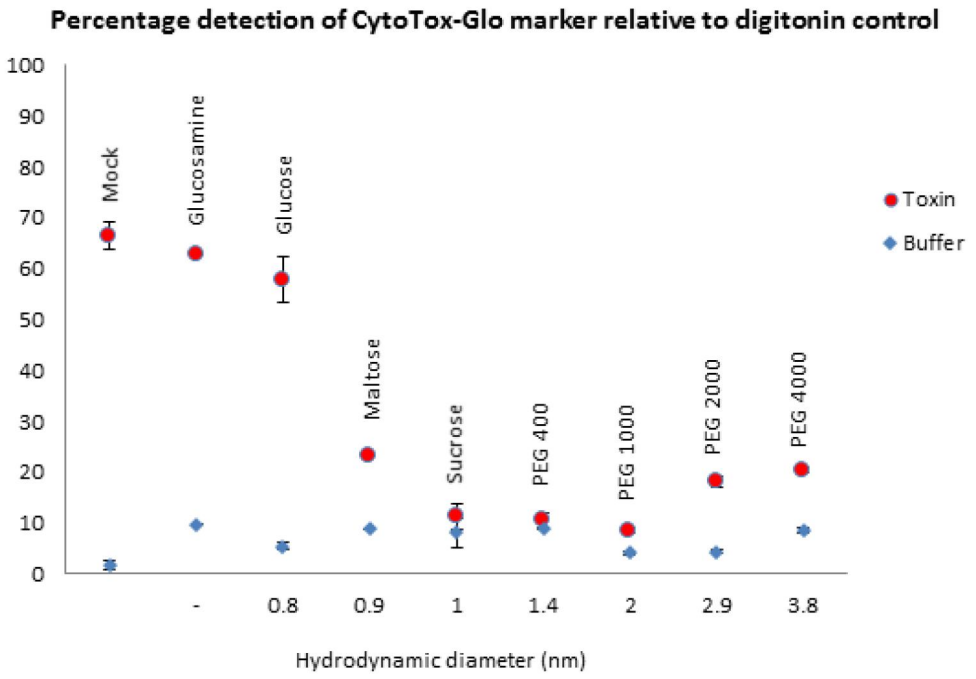

A Percentage ATP levels relative to buffer control
120
100
80
60
40
20
0
0.25
0.5
1
2.5
5.5
Time afer toxin exposure (hours)

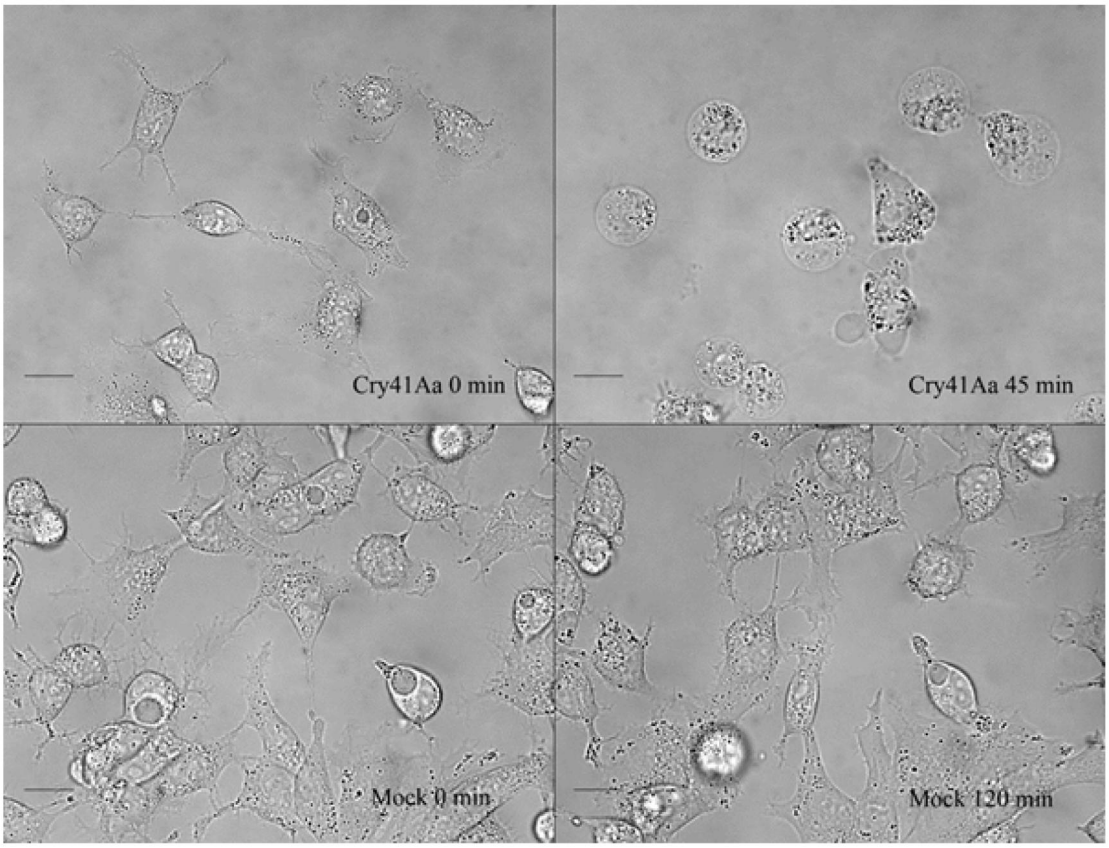

B Caspase levels (RLU)
160000
140000
120000
100000
80000
60000
40000
20000
0
2 hours
4.5 hours
7 hours
10 hours
24 hours
Etoposide
Cry1Ca
1µg/ml Cry41Aa
2µg/ml Cry41Aa
4µg/ml Cry41Aa
12µg/ml Cry41Aa
Mock


ROS levels (RFU)
5.0E+6
4.5E+6
4.0E+6
3.5E+6
3.0E+6
2.5E+6
2.0E+6
1.5E+6
1.0E+6
5.0E+5
0.0E+0
2 h
5 h
2 h
5 h
5 h
5 h
5
Cry41Aa (10)
Menadione
Cry1Ca (10)
Buffer
Digitonin


A CellTox Green cytotoxicity (RFU)
80000
70000
60000
50000
40000
30000
20000
10000
0
60 mins
90 mins
120 mins
150 mins
300 mins
Cry41Aa
Mock
Lysis Sol.


B Percentage membrane damage relative to digitonin control
80
70
60
50
40
30
20
10
0
15 min
30 min
1 h
2 h
3 h
4 h
5 h
6 h
6 h
6 h
6 h
6 h
6 h
6 h
5 (µg/ml)
5
58
5
58
Cry41Aa
Protoxin
Buffer
TX100
Cry1Ca


Percentage detection of CytoTox-Glo marker relative to digitonin control
100
90
80
70
60
50
40
30
20
10
0
Mock
Glucosamine
Glucose
Maltose
Sucrose
PEG 400
PEG 1000
PEG 2000
PEG 4000
Toxin
Buffer
-
0.8
0.9
1
1.4
2
2.9
3.8
Hydrodynamic diameter (nm)


Cry41Aa 0 min
Cry41Aa 45 min
Mock 0 min
Mock 120 min